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FEASIBILITY OF USING SALIVA AS A SCREENING SPECIMEN FOR DETECTION OF HUMAN CYTOMEGALOVIRUS(HCMV) IN NEWBORNS.

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ABSTRACT
Previous studies have shown that saliva is an important specimen for the diagnosis of congenital Cytomegalovirus (CMV). It is not only easy to collect without trauma to the newborn but neonates shed large volume of the virus in their saliva. Saliva which usually carry high viral load in infected patients especially in children, contain several components which inhibit the sensitivity of Polymerase Chain Reaction (PCR) in detecting CMV DNA. Two extraction methods; Guanidinium thiocyanate (GuSCN), Polyethylene glycol (PEG) were used. Both methods extracted CMV DNA at various degrees from saliva. Both PEG and GuSCN were efficient in removing salivary inhibitors in single individual saliva specimen than in pooled saliva probably due to more inhibitors in pooled saliva than in single saliva specimen. PEG was more efficient than GuSCN in the extraction of CMV DNA in pooled saliva. The PCR sensitivity achieved in single saliva specimen was ten-fold higher than in pooled saliva.

KEYWORDS: fetal abnormality, CMV infection, virus, baby infection, congenital transmission, fluids

INTRODUCTION

CMV is the leading cause of congenital infection all over the world (Haginoya *et al*., 2002) and it is the most common cause of fetal abnormality in developed countries (Chan *et al*., 2002, Selwyn, 2002). CMV infection is lifelong (Schleiss, 2005) and virus shedding following congenital, prenatal and postnatal infection lasts for many years (Stagno, 1995). In productive infection, virus is shed in blood, saliva, semen, cervical secretions, urine, tears, breast milk, amniotic fluid and infection arises following close contacts with these fluids (Stagno, 1995, Pass, 2001, Selwyn, 2002). HCMV transmission from mother to child is very common and this has become a major way of keeping the virus within the population (Pass, 2001). CMV congenital transmission is believed to be transplacental (Schleiss, 2005) and this occurs in women with primary and recurrent infection during pregnancy (Pass, 2001). A baby infected via this route will not manifest symptoms until at least 3 weeks after birth.

The morbidity associated with the infection in seronegative women who acquire the virus during pregnancy can be devastating to the foetus (Distefano *et al*, 2004). Some complications associated with congenital CMV infection include thrombocytopenia, hepatosplenomegaly, sensorineural deficits and mental retardation, spasticity, epilepsy and periventricular calcification, choroidoretinitis, vision loss and optic atrophy (Chan *et al*., 2002; Distefano, 2004). Rapid and accurate diagnosis of CMV infection is not only important in neonates where about 90% of those with congenital infection are asymptomatic at birth (Azam *et al*., 2001) but are also very important in immunocompromised and transplant patients especially in renal transplant where fatality is near 100% (Allen *et al*., 1994)

Endogenous reactivation of latent virus has been highly implicated in recurrent infection and appears to play a major role in intrauterine transmission during pregnancy in immune mothers (Stagno 1995). The rate of vertical transmission is between 20-40% in primary maternal infection which occurs in about 0.7-4.1% of women especially the unmarried young mothers of poor socioeconomic background during pregnancy (Pass and Boppana 1999; Chan *et al*, 2002). According to the work of Stagno *et al*., (1982), as cited by Pass (2001) about 2-28% of women below the age of 30 shed CMV in their vagina around the time of birth.

Series of diagnostic approaches have been used in the diagnosis of CMV infection. According to (Stagno, 1995, Nelson *et al*., 1995, Katz *et al*., 1998, Schleiss, 2005), isolation of the virus from urine or saliva within three weeks of birth is indicative of congenital infection after which it may be a result of exposure to vaginal secretion at birth (perinatal) or breast milk and blood products (postnatal).

The gold standard for conclusive diagnosis of congenital CMV is the isolation of the virus from cultures obtained from either the urine or saliva (Nelson, 1995; Pass, 2001; Schlesinger 2003), owing to the persistent excretion of the virus from the two sites (Tamura *et al*., 1980). However, the cultural isolation of the virus from urine in particular is regarded as a reference standard in diagnosis of congenital CMV (Balcarek *et al*., 1993) but many obvious reasons favour saliva as a screening specimen in children. Cytomegalovirus has predilection for salivary gland (Tamura *et al*., 1980) and this makes it an important specimen. Collection of saliva specimen is 'painless and non-invasive' (Ng *et al*., 2004). Others are the ease of specimen collection, absence of contamination often associated with urine, absence of nursing time to collect specimen, trauma and absence of specialized equipment (Warren *et al*., 1991; Victoria *et al*., 2004). Besides, collection of saliva specimen does not require specialized consumables like syringes and anticoagulants nor do they require special training (Ng *et al*, 2004). Further still, Ng *et al*., (2004) observed amongst other things that storing saliva for over one month does not affect DNA extraction, a finding which has been severally exploited in diagnosis. This appears to be most convenient in neonates and the very old because of the little or no complication and compliance difficulties associated with it (Kaufman and Lamster, 2002).

Though culture is a gold standard for CMV diagnosis, this process takes a long time and requires regular monitoring and maintenance of the media (Pass, 1995; Stagno 2001; Schleis, 2005). Even the modified, more rapid and sensitive vial assay which a is based on monoclonal antibodies also require multi-step processing and maintenance of the culture which produce low sensitivity when there is low viral titre (Victoria *et al.*, 2004). This cannot provide the quick and immediate result required in congenital CMV and in immunocompromised patients where such immediate and early diagnosis are essential for the management of the disease.

Due to the morbidity often associated with symptomatic congenital CMV infection and the sequeale of asymptomatic ones, a fast and rapid diagnostic test which is inexpensive, sensitive, specific and accurate is very important (Warren *et al*., 1991; Wang and Adler, 1996). PCR is proven to meet all these criteria with obvious advantages over culture (Revellow and Gerna, 2002). It is not only rapid, inexpensive and highly sensitive but little amount of the DNA of interest are specifically amplified and detected (Katz *et al.,* 1998). PCR has also been used to diagnose congenital CMV using different specimens. Allen *et al*., (1994) obtained 100% sensitivity in confirmed clinical isolates (29 of 29) and in culture positive urine specimen (10 of 10) using PCR-ELISA in congenital infected infants. Gouarin *et al*., (2002) used Real-Time PCR to quantify CMV DNA in aminiotic fluid of fetuses in which he established that CMV DNA is higher in symptomatic fetuses than in asymptomatic ones during intrauterine infection. Jones *et al*., (1999) also used Single-round PCR to establish the sensitivity and reliability of PCR in detecting CMV DNA in urine and amniotic fluid of congenital infected infants. Nelson *et al*., (1995) used PCR to diagnose congenital CMV in the serum of symptomatic congenital CMV infected children with 100% sensitivity and specificity. This also showed positive and negative predictive value of 100%. The result of the use of PCR in the screening of saliva specimen compared very well with tissue culture in sensitivity and specificity (Warren *et al.*, 1991). Warren *et al*., (1991) found 89% sensitivity and 96% specificity in relation to Tissue Culture (TC) in screening the saliva of 160 children between the ages of 1 and 14. They were of the opinion that this would be higher in newborns that are known to shed more viruses in saliva than the older ones. However, the major problem associated with the use of saliva as a screening specimen is the inhibition of the PCR sensitivity by some salivary products. These products include as cited by Behzadbehbahani *et al*., (1997), chelating agents such as EDTA that affect cations as Mg^{2+} whose activities are very important in the function of the *Taq* polymerase ,DNAse or RNAse that degrade DNA and RNA respectively, heparin, phenols, polyamins, polysaccharides calcium alginate. Others include, various proteins found in saliva, steroid hormones, immunoglobulin, insulin estradiols, drugs (Kaufman and Lamster, 2002). Notwithstanding the inhibition of this sensitivity by saliva, PCR are known to detect CMV DNA in saliva with high sensitivity and specificity. Various extraction methods have been used to improve on the sensitivity of PCR and each of them has removed these inhibitors to some degree (Behzadbehbahani *et al*., 1997). Various extraction methods have also been used to improve on the sensitivity of PCR, when saliva is used as a specimen in the diagnosis of CMV infection. Each of these extraction procedures has shown varying degrees of sensitivity depending on its ability to remove the inhibitors. Whole Saliva in addition to normal constituents of salivary origin, contain amongst others, serum and blood derivatives, gingival secretions and desquamated cells, cellular components, bacteria, food debris and drugs including the recreational drugs such as cocaine, opioids and others (Kaufman and Lamster, 2002).

This study aims at determining the suitability of saliva as a screening specimen for HCMV using PCR. Saliva specimen is easy to collect as it can be collected without trauma, contamination or special skills and training.

The sensitivity and specificity achieved will determine whether it can be employed to screen newborns for HCMV DNA in whom 90% are known to be asymptomatic in congenital infection. According to Behzadbehbahani *et al*., (1997) both GuSCN and PEG remove PCR inhibition to some extent.

MATERIALS AND METHODS

Saliva Samples

Saliva samples were collected from 50 M. Sc student volunteers in the Department of Medical Microbiology of the University of Manchester in the 2005/2006 academic session. Saliva samples were collected with sterile universal bottle and stored at 4^{o}C until use.

The saliva samples were pooled together and made into 50µl aliquots in sterile 0.5 ml ependorf tube. They were spiked with 50µl of AD169 CMV DNA at a concentration of 1.9 X 10^{6} pfu/ml from AD169 strain cultured in HEL cells in the virology laboratory of the Division of virology of the University of Manchester was used.

Method 1: Extraction with Guanidinium Thiocynate lysis buffer

4M solution of Guanidinium thiocynate (GuSCN) (Sigma Aldrich Ltd UK); 25mM Solution of sodium citrate (Sigma Aldrich UK Ltd); 0.5% w/v solution of N-Lauroylsarcosine (sodium salt) (Sigma Aldrich Ltd UK) final concentration; 1mM solution of Dithiothreitol solution (DDT) (Sigma Aldrich Ltd UK); 100µg/ml of glycogen (Roche Ltd UK) were added together in sterile distilled water (SDW) as the lysis buffer for each extraction carried.

In all samples including SDW used as negative extraction control, 200µl of the lysis buffer was placed in a sterile eppendorf tube and 50µl of the sample was added. This was properly mixed and incubated at room temperature for ten minutes. After the incubation, 25µl volume of 3M sodium acetate was added to give a final concentration of 0.3M. To this solution was added a further 275µl of cold iso-propanol which was mixed very well before incubating again at room temperature for another 10 min. The supernatant following centrifugation of the final solution for 10 min at 12000 x g rpm was decanted. The process was repeated with the pellet in 500µl of ice-cold ethanol. The pellet was allowed to air dry for 5 min and dissolved in 50µl of sterile distilled water.

Method 2 Extraction with Polyethylene Glycol

For each sample including SDW used as negative extraction control, 50µl of 30% w/v polyethylene glycol 8000 (PEG-8000) (Sigma Aldrich Ltd UK)was placed in a sterile eppendorf tube and 50µl of the sample added. To the mixture, was added 25µl of 3M sodium chloride (NaCl) (BDH Chemical Ltd UK) and mixed very well. This was then incubated on ice for 30 min. After the ice incubation, it was centrifuged at 10,000 x g for 15 min. The supernatant was decanted and the pellet re-suspended in 20µl of Tris-HCL (PH 7.0) (Sigma Aldrich Ltd) and incubated at room temperature for 10 min for use.

Polymerase Chain Reaction (PCR)

The following reagent volumes were mixed together in a 1.5ml eppendorf tube (Master Mix) in a DNA free room: 50µl of Buffer (10mM Tris-HCL. 50mM KCL, 2.5uM MgCl) (Roche Diagnostics Ltd UK); 5µl of 724c primer (0.2µM) (5'AAGAATCCTCACCTGGCTTTA3') (Bioline UK Ltd); 5µl of P2 primer (0.2µM) (5'TCGCTGTCTTCGACCGGTGA3') (Bioline UK Ltd); 10ul of deoxyribonucleotidetriphosphates (dNTP) (200µM) (Bioline UK Ltd); 2.5µl of amplitaq (1.25 units per reaction) (Roche Diagnostics Ltd); 377.5µl of sterile distilled water (ultra pure).

From this master mix, 45µl of the mixture was dispensed into PCR tubes. Ten-fold serial dilutions (Table 1) of the extracted sample (10^{-1} – 10^{-8}) were prepared in SDW and 5µl of each dilution added to the PCR reaction tubes in the DNA prep room. A 5µl volume of SDW was added to one of the tubes as a negative control (PCR control).The PCR amplification process was carried out in a Perkin Elmer 9600 version 3.01 automated thermal cycler. The amplification consisting of a single cycle involving denaturation at 94^{0}C for 10 min, annealing at 55^{0}C for 1 min and extension at 72^{0}C 1 min was followed by 40 cycles consisting of denaturation at 94^{0}C for 30 seconds, annealing at 55^{0}C for 30 sec and extension at 72^{0}C for 30 sec. Contamination was avoided by following strict laboratory and PCR protocols. 8% polyacriamide gel was prepared, poured and allowed to set in the gel mould by mixing the reagents above. 10ul of the sample was added to 1µl of loading buffer (40%w/v sucrose, 0.25%w/v bromophenol blue, distilled water) and loaded in 10ul volumes to the gel. A 1kb ladder was

also loaded (5µl volume) as a molecular marker in the gel. TBE (x1) buffer was used for the electrophoresis which was carried out at a constant voltage of 145v (398 mA).

At the end of the electrophoresis, the gels were removed, stained for 5 min in 1mg/ml ethidium bromide and destained in SDW for another 5 min. The stained PCR product (DNA) was visualised by placing in a UV transilluminator and polaroid pictures of the stained amplicons taken using Enhanced Analysis system (Herolab

Extraction of AD169 in pooled saliva using GuSCN and 724c/P2 PCR sensitivity

1 2 3 4 5 6 7 8 9 10 11

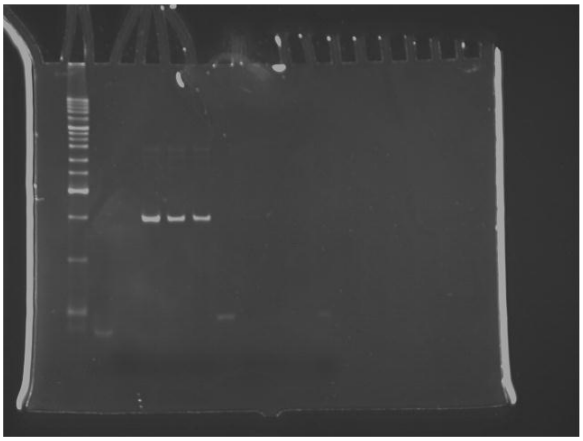

Figure 1: Serial dilution of AD169 CMV DNA extracted from pooled saliva using GuSCN and amplified with 724c/P2 PCR

1)Ladder/molecular marker, 2) Extraction control using SDW, 3) PCR negative control, 4)10^{-1} CMV DNA: positive, 5)10^{-2} CMV DNA: positive, 6) 10^{-3} CMV DNA: positive, 7) 10^{-4} CMV DNA: negative, 8) 10^{-5} CMV DNA: negative, 9) 10^{-6} CMV DNA: negative, 10) 10^{-7} CMV DNA: negative, 11) 10^{-8} CMV DNA: negative

Extraction of AD169 in individual Saliva using GuSCN AND 724c/P2 PCR sensitivity

1 2 3 4 5 6 7 8 9 10 11

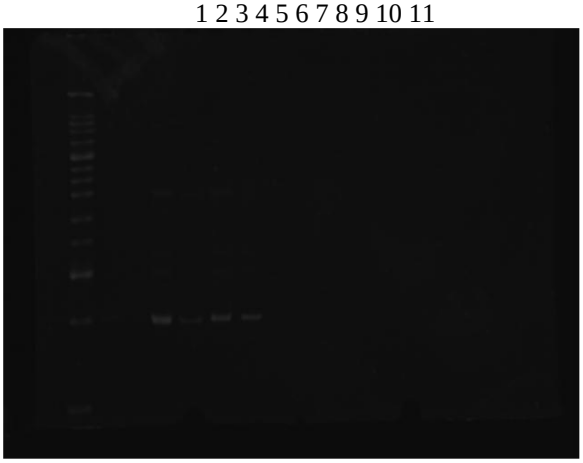

Figure 2: Serial dilution of AD169 CMV DNA in saliva extracted using GuSCN and amplified with 724c/P2 PCR

1) Ladder/molecular marker, 2) Extraction control using SDW, 3) PCR negative control, 4)10^{-1} CMV DNA: positive, 5)10^{-2} CMV DNA: positive, 6) 10^{-3} CMV DNA: positive, 7) 10^{-4} CMV DNA: positive, 8) 10^{-5} CMV DNA: negative, 9) 10^{-6} CMV DNA: negative, 10) 10^{-7} CMV DNA: negative, 11) 10^{-8} CMV DNA: negative

Extraction of AD169 CMV DNA from pooled saliva using Polyethylene glycol (PEG 8000) and 724c/P2 PCR sensitivity.

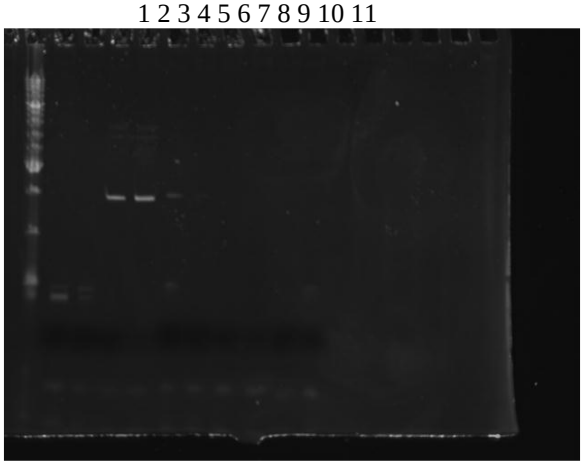

Figure 3: Amplification of AD169 CMV DNA extracted from pooled saliva using PEG with 724c/P2 PCR

1) Ladder/molecular marker, 2) Extraction control using SDW, 3) PCR negative control, 4)10^{-1} CMV DNA: positive, 5)10^{-2} CMV DNA: positive, 6) 10^{-3} CMV DNA: positive, 7) 10^{-4} CMV DNA: positive, 8) 10^{-5} CMV DNA: negative, 9) 10^{-6} CMV DNA: negative, 10) 10^{-7} CMV DNA: negative, 11) 10^{-8} CMV DNA: negative

Comparism of the PCR sensitivity of the extraction of AD169 DNA from saliva using various extraction methods.

Table 1: Dilutions at which the extraction methods were sensitive

DILUTION	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Pooled saliva extraction with GuSCN	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve
Individual saliva extraction with GuSCN	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve
Pooled saliva extraction using PEG	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve

DISCUSSION

In this study, two extraction methods were used to extract CMV DNA from pooled whole saliva of 50 student volunteers of the Department of Medical Microbiology of the University of Manchester. The methods used were Guanidinium thiocyanate and Polyethylene Glycol-8000. The two methods used were a modification of the method of Behzadbehbahani *et al.*, (1997). The concentration of AD169 used was 1.9 x 0^6 (pfu)/ml. When GuSCN was used to extract AD169 DNA from saliva, and ten-fold serial dilution of the CMV DNA amplified, the highest dilution at which the DNA was detected was 10^{-3} (Figure 1) for pooled saliva and 10^{-4} for individual saliva. When polyethylene glycol was used for the extraction of DNA from pooled saliva, 10^{-4} was the highest dilution that showed sensitivity (Figure 3). PEG extraction efficiency in pooled saliva was 10-fold higher than that obtained with standard GuSCN which collaborated with the finding of Behzadbehbahani *et al,* (1997) that PEG was a more effective extraction method. When GuSCN was used for DNA extraction in a single saliva, the

sensitivity was ten-fold 10^{-4} (Figure 2) higher than the result obtained from pooled saliva 10^{-3} (Figure 1) This was thought to be either due to higher level of inhibitors in the pooled saliva or some of the volunteers might have had diets, drugs and other inhibitors that are not the normal constituents of saliva. It could also be both.

The advanced reasons was suspected to have increased the inhibitory capability of the pooled saliva impacting on the efficiency of the extraction and hence the sensitivity of the PCR. The single individual saliva does not contain the inhibitors found in the pooled saliva which affected the efficiency of the extraction method.

In conclusion, PCR detected CMV DNA in saliva samples to a high degree of specificity (band of 300 bp) and sensitivity. The sensitivity achieved was a direct result of the efficiency of the extraction method. It was also found that PEG as a method was easier to prepare and run and hence could easily be used in large population studies, a finding in conformity with Behzadbehbahani *et al* (1997). Polyethylene glycol is a non toxic water soluble reagent as reported by Behzadbehbahani *et al* (1997) and this makes it very safe for use unlike GuSCN which is highly toxic.

In this study, it was observed that GuSCN was more efficient in non pooled saliva probably because it lacked some inhibitors, which must have been present in the pooled saliva. Going by this finding, it is most probable that both methods will show higher extraction efficiency and PCR sensitivity in saliva of neonates as the lack most of the inhibitors found in adult saliva used in this research. The only diet of neonates are breast milk and water as the case maybe and this rules out diet associated inhibitors found in saliva of adults such as drugs that reasonable increase their inhibition (Kaufman and Lamster, 2002). If GuSCN whose efficiency was ten-fold lower than that obtained by PEG in pooled saliva showed high sensitivity in individual saliva, then PEG will show far more sensitivity when applied to individual saliva as well as saliva of the newborns. Neonates are immunologically naive and therefore their saliva lack lots of proteins, immunoglobulin and antibodies found in adult saliva which inhibits PCR efficiency. Consequently, neonatal saliva holds a promise as an alternative potential clinical specimen in screening for congenital CMV in newborns. A follow up with actual use of saliva of neonates is suggested. The need for an easy, rapid and efficient extraction method is very essential if the sequeale that come along with congenital CMV infection is to be properly diagnosed, monitored and managed. Further studies should be carried out to find more ways of removing PCR inhibitors in saliva as that will not only increase the reliability of saliva as a diagnostic specimen in neonates but in all cases of CMV infection.

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